JOURNAL OF CHEMICAL EDUCATION

Laboratory Experiment

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A Sensitive and Robust Enzyme Kinetic Experiment Using Microplates and Fluorogenic Ester Substrates

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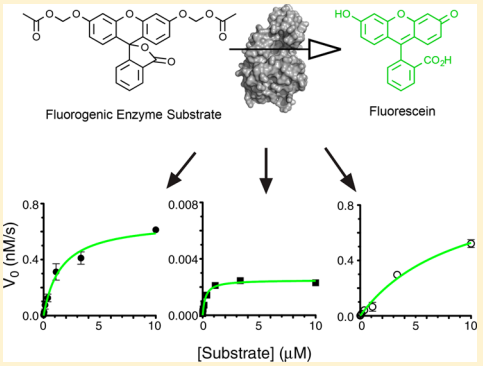
Supporting Information

ABSTRACT: Enzyme kinetics measurements are a standard component of undergraduate biochemistry laboratories. The combination of serine hydrolases and fluorogenic enzyme substrates provides a rapid, sensitive, and general method for measuring enzyme kinetics in an undergraduate biochemistry laboratory. In this method, the kinetic activity of multiple protein variants is determined in parallel using a microplate reader, multichannel pipets, serial dilutions, and fluorogenic ester substrates. The utility of this methodology is illustrated by the measurement of differential enzyme activity in microplate volumes in triplicate with small protein samples and low activity enzyme variants. Enzyme kinetic measurements using fluorogenic substrates are, thus, adaptable for use with student-purified enzyme variants and for comparative enzyme kinetics studies. The rapid setup and analysis of these kinetic experiments not only provides advanced undergraduates with experience in a fundamental biochemical technique, but also provides the adaptability for use in inquiry-based laboratories.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Inquiry-Based/Discovery Learning, Enzymes, Kinetics, Proteins/Peptides, Fluorescence Spectroscopy, Microscale Lab*

Michaelis–Menten enzyme kinetics is the pinnacle of many first-semester biochemistry courses and a central topic in biochemistry laboratory education.[1,2] Laboratory procedures for measuring enzyme kinetics, published in this Journal and elsewhere, diverge based on enzymes tested, methods of detection, experimental throughput, and data analysis methods.[3−9] Serine hydrolases, including esterases, lipases, and phosphatases, have been a common theme of undergraduate enzyme kinetic experiments.[3,4,6,8,9] With their well-understood enzyme mechanisms, commercially available substrates, and interesting biotechnological and therapeutic applications, serine hydrolases have served as the centerpiece of biochemistry, biophysics, organic chemistry, and general chemistry laboratory procedures.[3,4,6,8−12]

Enzyme kinetic experiments with serine hydrolases have also been adapted to the ongoing trend of microplate analysis in the undergraduate laboratory.[3,4] The miniaturization of kinetic experiments with microplates increases the speed of data acquisition and introduces students to high-throughput methodology.[3,5,13,14] Although microplate instruments are more expensive, the overall cost of conducting the experiment is decreased because of reduced sample sizes, the necessity for only a single instrument to serve a relatively large number of students rapidly, and a lower chemical input.[5,15,16] Many common kinetic assays are, however, not amenable to microplate format as they lack the sensitivity, reproducibility, and stability to be scaled to microplate format. To compensate for this lack of sensitivity, previous microplate-based experiments have utilized commercially available enzymes at high concentrations.[3,4] These assays are, however, incompatible with inquiry-based experiments using student-prepared enzyme samples, where enzyme samples are only prepared in fairly low concentrations. Experiments on comparative enzyme kinetics also require that students measure the kinetics of low activity enzyme variants with substitutions in key catalytic and binding pocket residues, which cannot be completed with current microplate experiments because of the lack of sensitivity.

Herein, a laboratory experiment is outlined for the microplate analysis of student-designed serine hydrolase variants. This laboratory experiment utilizes fluorogenic ester substrates to increase the sensitivity and throughput of undergraduate student kinetic analysis. The relevant benefits and chemical properties of these fluorogenic ester substrates are provided in the Supporting Information. This straightforward enzyme kinetic assay has provided reproducible student kinetic data in triplicate for over 50 upper-level undergraduate biochemistry students against four different serine hydrolases. The major advantage of this redesigned kinetic laboratory is its adaptability and sensitivity for use in an inquiry-based experiment using student-produced enzyme variants.

dx.doi.org/10.1021/ed500452f | J. Chem. Educ. XXXX, XXX, XXX−XXX

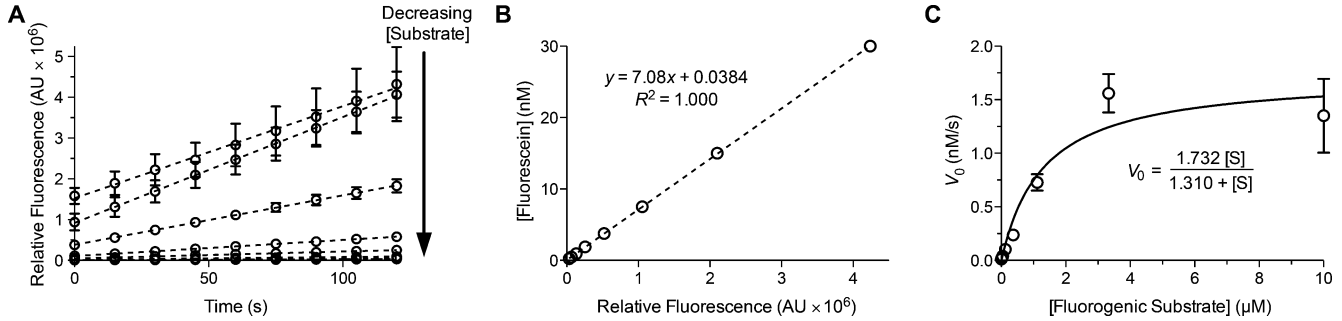


Figure 1. Basic student enzyme kinetic data and kinetic analysis. (A) The fluorogenic ester substrate was serially diluted eight-fold with PBS buffer and incubated with the bacterial hydrolase TM0077 from *Thermotoga maritima*.[18] The increase in fluorescence intensity over time was linear and correlated with the concentration of substrate added to the reaction. (B) The relative fluorescence values were converted to molar concentration using a fluorescein standard curve. (C) Average initial velocities were fitted to the standard Michaelis–Menten equation of V_0 (initial velocity) versus fluorogenic substrate concentration by nonlinear curve fitting, and values for the kinetic constants (V_{max}; K_M) were extracted. For wild-type TM0077, the kinetic constants were k_{cat} = 0.044 ± 0.005 s^{-1}, K_M = 1.3 ± 0.5 μM, and k_{cat}/K_M = 3.4 × 10^4 ± 1.4 × 10^4 M^{-1} s^{-1}.

LEARNING OBJECTIVES

From this experiment, students are introduced to the principles of enzyme kinetics including the importance of measuring initial rates, fitting data to the Michaelis–Menten equation, extracting values for kinetic constants, and methods for obtaining reproducible enzyme kinetics. Students also learn high-throughput methodology, especially microplate analysis, serial dilutions, and the use of multichannel pipettors. On the basis of the comparison of the relative enzymatic activity, students also gain experience into comparative enzyme kinetic analysis and understanding of enzyme structure–activity relationships.

EXPERIMENTAL PROCEDURE

Within an upper-level, inquiry-based biochemistry laboratory, students design and construct variants of a bacterial hydrolase, purify their protein variant to homogeneity using nickel affinity chromatography (80–95%), and characterize the purity and basic biochemical properties.[17] The entire laboratory sequence involves 10 weeks: 4 weeks for students to construct their hydrolase variants, four more weeks to purify and confirm their variants, and finally 2 weeks for kinetic analysis of the variants (the subject of this article). In different semesters of the laboratory, students have explored the kinetics of diverse bacterial hydrolases (EC: 3.1) including *Tm0077* (an acetylesterase from *Thermotoga maritima*), ybfF (a thioesterase from *Vibrio cholerae*), Rv0045c (a carboxylesterase from *Mycobacterium tuberculosis*), and LipW (a lipase from *Mycobacterium tuberculosis*).[18,19]

The experimental portion of the kinetic experiment is conducted in one, 4 h laboratory period, and analysis of the kinetic data is conducted in the next laboratory period. The experimental portion is broken up into two main sections: the preparation of serial substrate dilutions and the assembly of the microplate for analysis. The serial dilution series results in eight different substrate concentrations across an overall 2000-fold concentration range. Multichannel pipettors are used for this serial dilution to provide practice with multichannel pipettors and to allow all serial dilutions to be completed in parallel. In addition to the substrate dilutions, dilutions of enzyme samples (student hydrolase variants (2x) and wild-type hydrolase (1x)) and of fluorescein standards are also prepared. After combination of substrate and enzyme dilutions on the assay plate, the actual kinetic assay takes only 4 min for each plate, which allows all eight classroom students to collect kinetic data for their individual three enzyme variants on one instrument in less than 4 h. At the beginning of the next laboratory section, students analyze their enzyme kinetic data using an instructor-provided spreadsheet to assist in the kinetics calculations (Supporting Information). In the end, students fit their kinetic data to a standard Michaelis–Menten equation and extract values for V_{max}, K_M, k_{cat}, and their respective errors. A detailed version of the experimental procedure along with a detailed diagram of the setup for the microplates are provided in the Supporting Information.

HAZARDS

Fluorogenic ester substrates are dissolved in dimethyl sulfoxide. Dimethyl sulfoxide can be readily absorbed through the skin and is a potential irritant. Gloves and proper eye protection should be worn while handling the substrates in DMSO. Avoid inhalation of lyophilized acetylated BSA, as the lyophilized powder is a potential irritant. All other chemicals for this lab are aqueous buffers, salts, and low concentrations of proteins in aqueous solution with minimal hazards and should be handled with standard laboratory safety procedures including proper eyewear, lab coats, and gloves.

RESULTS AND DISCUSSION

In a one-semester biochemistry laboratory course, students purified unique variants of different bacterial hydrolases and then determined the biochemical characteristics of their protein variants.[17] One biochemical characteristic that students measured was the differential enzymatic activity of their protein variants in comparison to the wild-type enzyme. For enzymatic activity, simple fluorogenic enzyme substrates were used that allowed students to measure reproducible, accurate kinetics for even low activity enzyme variants (Figure 1). This laboratory experiment has been conducted five times with over 50 undergraduate students studying four different bacterial hydrolases.

For the assay, the fluorogenic enzyme substrates remained nonfluorescent until addition of the hydrolase immediately before analysis. The hydrolase then rapidly removed the ester protecting groups and exposed the highly fluorescent free fluorescein.[20] The rate of fluorogenic substrate activation was measured over a range of substrate concentrations (Figure 1A), and the relative fluorescent units were converted to molar concentrations using a fluorescein standard curve (Figure 1B).

dx.doi.org/10.1021/ed500452f | J. Chem. Educ. XXXX, XXX, XXX–XXX

By performing the reaction in triplicate at a range of substrate concentrations, students constructed a plot of substrate concentration versus initial velocity for their hydrolase variants. The kinetic data were then fitted to the Michaelis–Menten equation to extract values for kinetic constants (V_{max}, K_M, k_{cat}, and k_{cat}/K_M) (Figure 1C).

For the biochemistry course, the same protein variants were assigned to two different students, which allowed direct comparison of their kinetic results from two separate enzyme preparations. Overall, student kinetic data with the fluorogenic substrates have been reproducible and well described by the Michaelis–Menten equation (Figure 2). Although the V_{max} values for the student curves shifted slightly, the overall structure of the Michaelis–Menten curves and the K_M values remained fairly constant (Figure 2 and Table 1). The variation in V_{max} values, but not the K_M values, suggested that the variation between student data originated from variations in enzyme concentrations in the reaction. This discrepancy in enzyme concentrations could be due to random errors, such as inconsistent enzyme concentration due to a lack of prior experience with the multichannel pipets, or to systematic errors such as impurities in student-purified enzyme samples. Errors for the kinetic constants based on the triplicate kinetic data were, however, within an acceptable range for publishable kinetic data, which confirms one of the learning objectives (Table 1).[18,19] Comparison of student kinetic results also illustrated the ability of laboratory students to measure the enzymatic activity of hydrolase variants with diverse kinetic activity (Figure 2 and Table 1). Even variants with low enzymatic activity provided kinetic constants and error values above the background for the measurement (Table 1).

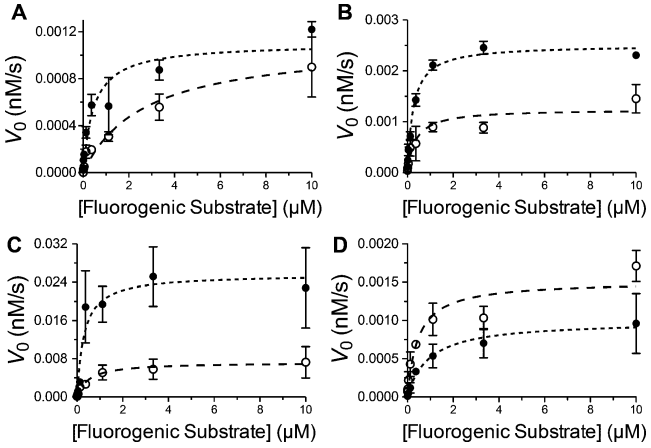


Figure 2. Comparative student kinetic data for four different enzyme variants. Each plot shows the Michaelis–Menten curves of the same enzyme variant purified and tested by two different students. Kinetic values from each curve are given in Table 1. The first two plots used the TM0077 enzyme from *T. maritima*, and the second two plots used the ybfF enzyme from *Vibrio cholerae*.[18,19] (A) Y86A TM0077. (B) I276A TM0077. (C) M87A ybfF. (D) Y209A ybfF.

Table 1. Kinetic Comparison for Individual Student Data in Figure 2

Panel/Student[a]	k_{cat} (10^{-3} s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
A/1	0.028 ± 0.003	0.46 ± 0.17	61 ± 23
A/2	0.027 ± 0.005	2.7 ± 1.3	10 ± 5
B/1	0.064 ± 0.002	0.26 ± 0.03	250 ± 30
B/2	0.032 ± 0.004	0.35 ± 0.17	91 ± 45
C/1	0.13 ± 0.02	0.30 ± 0.17	430 ± 250
C/2	0.036 ± 0.006	0.50 ± 0.33	72 ± 49
D/1	0.0075 ± 0.0007	0.46 ± 0.17	16 ± 6
D/2	0.0050 ± 0.0008	0.93 ± 0.55	5.4 ± 3.3

[a]Kinetic constants were determined by measuring the increase in fluorogenic enzyme substrate fluorescence over time. Data were fitted to a standard Michaelis–Menten equation to determine the values for k_{cat}, K_M, and k_{cat}/K_M. Kinetic measurements for each substrate were repeated three times, and the values are given ± standard error (SE).

On the basis of the differential kinetic constants of their hydrolase variants and the three-dimensional structure of the hydrolase, students constructed a hypothesis about the role of their amino acids in determining the enzymatic activity of the hydrolase. This analysis was inserted into a written report where students presented tables of their kinetic results, Michaelis–Menten plots for their kinetic data, and structures of the hydrolase-binding pocket supporting their hypothesis. These reports were used to assess the major learning objectives about the analysis of enzyme kinetics and the construction of enzyme structure–activity relationships. On the basis of five years of student data and written student reports, students were highly successful with providing correct explanations for residues directly involved in catalysis (catalytic triad; oxyanion hole) but struggled more with activity changes based on small structural differences in binding pocket residues such as variations in hydrophobicity and steric hindrance.

A potential drawback to this methodology is that the fluorogenic substrates are not commercially available. Undergraduate chemistry students in a separate class, however, prepared some of the fluorogenic substrates for the laboratory by following published procedures.[18−21] The low concentration of fluorogenic substrate required for each reaction allowed a single milligram-scale batch of synthetic substrate to be used for five years of the course. The ease of the synthesis of the fluorogenic substrates has also allowed small modifications of the ester functionalities on the substrates to accommodate variations in the substrate specificities of chosen bacterial hydrolases.[19] A small batch of fluorogenic substrate can also be provided to interested instructors to facilitate introduction of the laboratory procedure. Finally, the experimental procedure has been adapted to commercially available *p*-nitrophenyl substrates with modifications to correct for the decreased stability and sensitivity of these substrates (Supporting Information).

■ CONCLUSIONS

Overall, this experiment, which combined fluorogenic enzyme substrates, microplates, and high-throughput methodology, provided the versatility, sensitivity, and speed necessary to be used as a general method to measure enzyme kinetics in an undergraduate biochemistry laboratory course. The substitution of the fluorogenic enzyme substrates in place of traditional ester substrates provided lower background signal and higher sensitivity, which allowed students to measure reproducible enzyme kinetics with even low activity student-purified enzyme variants. The increased accessibility of microplate readers and their adaptability to low protein volumes and sample input allowed standard enzyme kinetic measurements to be redesigned for an inquiry-based biochemistry laboratory. Kinetic measurements using fluorogenic ester substrates were also adaptable to a variety of student classroom projects or

dx.doi.org/10.1021/ed500452f | J. Chem. Educ. XXXX, XXX, XXX−XXX

expandable into further experiments on enzyme kinetics as described in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

Instructor notes with possible additional experiments; student notes; kinetic analysis help sheet; and adaptation of the laboratory procedure to *p*-nitrophenyl substrates. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

R.J.J. and G.C.H. gratefully acknowledge support for the laboratory from a National Science Foundation grant (DUE-1140526). The authors are grateful to the students from course CH463 2010−2014 for piloting the kinetic activity measurements. The authors also thank Hai Pang (Tsinghua University) who kindly provided the expression plasmid for Rv0045c. L.D.L. acknowledges support by the Howard Hughes Medical Institute.

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